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Magnetic Microcapsule Exploration in the Gastrointestinal Cavity of the Origins of Colorectal Cancer-Associated DNA-Damaging Agents in the Human Diet

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Magnetically recoverable, semipermeable microcapsules have been devised for covalent entrapment of reactive substances in the intestinal cavity to biomonitor potentially DNA-damaging agents and the effects of etiologically important components of the human diet. These microcapsules have been shown to trap five types of agents *in vivo*, namely, carcinogen electrophiles, nitrosating agents, mutagens/carcinogens having a planar molecular structure, and as-yet unidentified endogenous cross-linking agents and precursors of reactive oxygen species. Substantial alterations in both total metabolites and types of metabolites trapped from [^{14}C]benzo(*a*)pyrene were found to be caused by increasing (within the human intake range) the dietary levels of beef protein and dietary fiber. The system thus responds to a variety of potentially critical agents and in a manner consistent with epidemiologically important dietary modulators for colorectal carcinogenesis. Work toward recognizing entrapped endogenous agents has also begun.

Introduction

Because the human diet (*1*) and gastrointestinal (GI) sites (*2*) have the major nontobacco roles in human carcinogenesis, the general aim of our work has been the development and use of a system capable of selectively trapping otherwise inaccessible DNA-damaging agents in the human GI tract. To facilitate the short-term molecular identification of GI carcinogens and their dietary sources, our work has involved devising not only a means of monitoring the lower GI tract but also methods to independently test the influences of single components of human diets on (model) carcinogens within the GI cavity. The work reviewed here is the development of recoverable microencapsulated targets (*3*), their applications in the use of carcinogens and precursors, the first use of human diets altered systematically in colorectal (CR) cancer risk components (*4,5*), and an introduction to the first use of these microcapsules in humans, which is reported in detail elsewhere in this issue (*6*).

Since the inconclusive 1987 review by Bruce (*7*) on postulated determinants of CR cancer, a series of important findings have been made: *a*) *ras* oncogene activation and *p53* inactivation were found, respectively, in half or most of CR cases (*8*); *b*) different acetylator status was found for CR cases (*9*); *c*) the principal fecal mutagen (fecapentaene) was demonstrated as a rodent carcinogen (*10,11*) and CR cases were found to have a 6-fold lower stool residual concentration of fecapentaene than controls (*12*); *d*) dietary iron and phytate, respectively, were shown to enhance or decrease 1,2-dimethylhydrazine (DMH)-induced tumorigenesis (*13,14*), and human feces were shown to contain very high levels of hydroxyl radical generators (*15*), all in accord with the hydroxyl radical hypothesis of Graf and Eaton (*16*); *e*) the protein kinase C activator diacylglycerol was found to be produced by intestinal microflora (*17*); and *f*) several studies in humans confirmed the CR-protective effects of bulking fiber, antioxidant vitamins, and calcium (*18–20*). The last four findings involving substances or sources passing through the GI cavity (together with classical studies on dietary/microfloral manipulation of experimental CR carcinogenesis) indicate that the GI cavity is the principal source of CR carcinogenic agents.

Although entrapment within the GI cavity could reveal substances of interest, the three following conundrums nevertheless had to be faced: *a*) If we do not know what types of substances are to be trapped, what type of trapping system should be used? *b*) Since many dietary interactions are already known, yet complete human diets typical of human everyday consumption have never been used in experimental animals, how can we quantify and control the probable (major?) influences on microcapsule trapping of model carcinogens by dietary risk factors already shown by epidemiological means? *c*) Since GI transit time is typically several days and dietary intervention studies require at least several weeks, how can short-term monitoring be used

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to approach long-term, multistage carcinogenesis? We are considering here our most extensive environmental exposure that occurs whether or not the individual has other lifestyle or workplace exposures, and for which highly developed techniques have long been used in clinical nutrition studies. The GI milieu requires only a few transit times to adapt to a chosen diet and to produce new agents of interest, and this must not be confused with the months of dietary intervention needed to observe changes in, say, cellular proliferation rates. Hence the briefness both of adaptation and of microcapsule exposure provides realistic opportunities to correlate the changes in what microcapsules trap with a series of systematic changes in diet; CR risk factors established already through long-term epidemiological studies and medium-term intervention studies can be used as benchmarks.

Basic Features for the Monitoring System

Excretable Microcapsules within the GI tract

The basic idea is to orally administer millions of small microcapsules that disperse within the GI tract and collectively provide a large surface area for trapping reactive substances that probably would not be excreted into the feces. Subsequent fecal excretion of the microcapsules with reactive agents trapped and protected within the microcapsule interior should preclude loss of reactive agents (Table 1) by mucosal absorption or microfloral metabolism.

Preparation and Basic Properties of Microcapsules As Traps for Alkylating Agents

Although microencapsulation has permitted the controlled release of pharmaceuticals, pesticides, and other agents through the destruction of the membrane encapsulating the active principal, we needed to produce microcapsules that are stable and selectively entrap low molecular weight substances that enter through a semipermeable membrane (*3*). Several major advantages are realized with a membrane that both excludes enzymes, bacteria, and macrophages, which could destroy the entrapped carcinogen, and also retains a water-soluble macromolecular target inside the microcapsules. Because most covalent-binding reactions of DNA (not necessarily those most important in causing miscoding) are on the nitrogen atoms of guanine and adenine, we used polyethyleneimine (PEI) as a first approach to a DNA surrogate. For simple use, we arranged a quick method of recovery from feces by including magnetite in the microcapsules. There is substantial art in producing microcapsules and minor charges in production conditions commonly give inexplicable and profound alterations to microcapsule batches; nevertheless, the conditions were optimized for membranes of poly (hexamethylene-terephthalamide) grafted onto PEI so as to enable trapping of [$^{14}CH_3$]-*N*-nitrosomethylurea (NMU) *in vivo* (*21*,*22*).

Several key features were demonstrated during the early work: *a*) microcapsules were capable of entrapping substances up to a molecular weight of about 1000 *in vitro*; *b*) use of hemoglobin instead of PEI resulted in microcapsules that were proteolytically unstable, presumably due to hemoglobin molecules within the membrane being available to enzymic attack; *c*) rats showed no evidence of distress or harm from gavage treatments with the microcapsules; *d*) microcapsules could be recovered intact by simple magnetic means from the feces; *e*) the microcapsules could be broken ultrasonically after treatment *in vivo* with ^{14}C-labeled NMU or methyl iodide, to show a core versus membrane distribution of PEI; *f*) which in turn was radically altered by changes in membrane formulation; *g*) microcapsules after GI transit unexpectedly became stronger (more resistant to ultrasonic rupture).

In another study (*23*), entrapment *in vivo* was shown for an IP-administered carcinogen requiring metabolic activation, dimethylhydrazine (DMH). The time course of trapping showed that microcapsules administered more than 2 hr after injection of DMH trapped relatively much less of the DMH dose presumably because they were not in position for biliary-excreted metabolites. For [^{14}C]NMU administered intrarectally, only microcapsules given by gavage within the time 4 to 8 hr before showed any trapping. That is, the microcapsules had to reach the rectum to be in position to intercept the electrophilic species

Table 1. Substances detected in GI cavity by IARC microcapsules.[a]

Reactant	*In vitro*	*In vivo*	Diet effect	Microcapsule type[b]	Assay type[c]	Substances
Direct-acting carcinogen electrophiles	x	x	–	1,2,3	RA	NMU, MMS, CH_3I
Carcinogen with host activation	–	x	x	1,2,3,4	RA	IQ, BaP, PhIP DMH
Planar structure carcinogen	x	x	–	1,4	RA, C	IQ,BaP, PhIP, Glu-P-1, endogenous
N-nitrosating agents	x	x	–	1,5	TEA	Nitrite/drinking water
Cross-linking agents	x	x	x	4,5	M/T	Many, endogenous
Reactive oxygen species and precursors	x	x	x	5	RA	H_2O_2, endogenous

Abbreviations: NMU, *N*-nitrosomethylurea; MMS, methylmethane sulfonate; IQ, 2-amino-3-methylimidazo[4,5-*f*]quinoline; BaP, benzo[*a*]pyrene; PhIP, 6-phenyl-2-amino-1-methylimidazo[4,5-*f*]pyridine; DMH, dimethylhydrazine; Glu-P-1, 2-amino-6-methyldipyridol[1,2-*d*]imidazole; endogenous, as-yet unidentified endogenous agents.

[a](x) Successful demonstration, (–) not demonstrated.

[b] Microcapsule types were 1, PEI; 2, poly(vinyl alcholol triethyleneteramine) (PVA-TET); 3, PVA-TET acetylated; 4, PEI CPTS; and 5, $^{14}CH_3$-labeled PEI.

[c]Assay types were RA, radioactive counting; C, HPLC; TEA, thermal energy analyzer; M/T, large alteration to ratio membrane/total label distribution.

from the fast-decomposing NMU. They were still able to perform such trapping after hours of admixture with intestinal contents.

A more detailed investigation (*24*) in trapping [^{14}C]benzo[*a*]pyrene (BaP) metabolites showed that a single dose of BaP given by gavage yielded trappable metabolites mostly in the first 48 hr. The pattern of ^{14}C activity in an extraction sequence (A, methanol-NH_4OH desorption; B, acid hydrolysis; C, solvent extraction; D, inextractable) was unlike either that from simple mixing of microcapsules with feces containing BaP metabolites or *in vitro* treatment with 7,8-dihydroxy,-9,10-epoxy-7,8,9,10-tetrahydrobenzo(*a*)pyrene. Of the HPLC-identified metabolites, the 1,6- and 3,6-diones were predominant, and desorbable metabolites were mostly unconjugated.

A further study (*25*) with the protein pyrolysates 2-amino-3-methylimidaza[4,5-*f*]quinoline (IQ) and 6-phenyl-2-amino-1-methylimidazo[4,5-*f*]pyridine (PhIP) has shown both substances to be trapped *in vivo*. Thus, for all alkylating agents administered by different routes, trapping within the GI cavity was demonstrated provided that the microcapsules were present at a time when electrophilic products could be expected to be present in the local GI segment. The products entrapped are thereby derived from a brief exposure period, and this feature has the potential for providing a better temporal discrimination of their sources than, say, long-term integration by adducts on blood proteins.

Endogenous Cross-Linking and Nitrosating Agents

Further investigation of the surprising finding that microcapsules became stronger in the GI tract concluded that this was caused by cross-linking of available PEI amines, leading to both intramembrane reactions and core-membrane cross-linking (*26*). Such effects could be produced *in vivo* with recognized cross-linking agents, and with fecapentaene-12 and 4-hydroxynonenal, which are of interest as potential endogenous carcinogens. This work showed that endogenous cross-linking agents are present extensively within the rat GI tract and were present in both gastric and large bowel sections of chow-consuming F344 rats. These effects were readily detected by measuring the core-to-membrane shift of $^{14}CH_3$ label in PEI microcapsules, yielding acid-resistant cross-links in the large bowel. A recent study has shown the presence of extensive cross-linking agents presence in humans (*6*), with even larger effects obtained by 48 hr anaerobic incubation with fecal slurries. Cross-linking agents are inherently of considerable interest, as they would seem to be bifunctional alkylating, membrane-penetrating agents. Many cross-linking agents are carcinogens (*27,28*), able to activate *fos* oncogene (*29*), and are particularly potent mutagens in *Drosophila* (*30*). Although we are not yet able to identify them (indeed there is no other detection system for cross-linking agents), their presence deserves further investigation.

The detection of endogenous nitrosating agents through urinary *N*-nitrosoproline excretion (*31*) initiated many studies in this field. Because the pK_a of the PEI homologue piperazine renders it much more susceptible to nitrosation than the PEI homologue of proline (*32*), the nitrosation of PEI microcapsules was studied (*33*). The capsules were found to be nitrosated with a pH profile similar to amides rather than amines, which was of interest because the major nitrosatable substrates at gastric pH are believed to be amides (*34*). *In vivo* nitrosation was demonstrated (*33*) and preliminary results suggest that they are much more effective in trapping nitrosating agents than proline.

Mutagens and Carcinogens with Planar Molecular Structures

In examining the trapping of [^{14}C]BaP *in vivo*, it was found (*24*) that about 25% of total radioactivity could be desorbed with the methanol-NH_4OH mixture used for extracting adsorbed mutagens/carcinogens with a planar molecular structure from blue cotton (*35*). Because blue cotton cannot be readily ingested, we covalently modified PEI microcapsules with the copper phthalocyanine tetrasulfonic acid moiety (CPTS) used in blue cotton. The resulting blue–green microcapsules have shown an enhanced propensity to reversibly absorb both BaP (*36*) and the protein pyrolysates IQ, PhIP, and 2-amino-6-methyldipyridol-[1,2-α:3:2.-*d*]imidazole (Glu-P-1) (*25*). These were shown to have a lower net trapping of BaP and its metabolites *in vitro and in vivo*, but a higher proportion of the total could be desorbed. These results are of interest because several large groups of carcinogens, including some causing intestinal tumors, have planar structures.

Effects of Ingestion of Microcapsules in Rodents

Several studies were undertaken to demonstrate lack of harm; it should be noted that the human GI tract is exposed to huge numbers of particles of the size range of our microcapsules both from dust on vegetables and following the mucociliary clearance from the lungs of inhaled particles. Following five doses of microcapsules, F344 rats showed the same mortality pattern during 120 weeks as historical controls (*4*). No translocation out of the GI tract and a 98.7% recovery of radioactivity were found for rodents treated with radiolabeled microcapsules (*37*). Chronic dosing caused no retention in GI structures as seen when examined microscopically.

Presenting DNA-Type Targets to Identify and Discriminate Important DNA-Damaging Agents and Their Sources

In our approach to the long-standing general problem of how to trap and determine the structure of as-yet unidentified DNA-damaging agents (*38*), we considered the following three limitations: *a*) the only highly sensitive chemical assay procedures that can provide structural information is mass spectrometry; *b*) numerous biological/biochemical assay systems can indicate that DNA damage has taken place, but cannot specify what caused the damage; *c*) it can be expected that a large proportion of DNA-damaging agents give no tumorigenic or mutational outcome, so that some discrimination is needed. Therefore, as our eventual purpose is to identify dietary risk factors that must be avoided, the choice of both target and assay techniques for substances trapped by microcapsules should provide both biological relevance and best chemical specificity for attribution to individual sources. Thus, two types of trapping agent or target are needed; one of low molecular weight that can enable identification for at least several types of adducting substance by MS and/or ^{32}P-

Table 2. Effects of independently altered human dietary macrocomponents of fat, beef protein, and fiber nonstarch polysaccharide on microcapsule trapping, colonic nuclear aberrations, and BAP 1,6-diaone trapping arising from treatment with BaP.[a]

	C57/B6 mice		F344 rats	
Fat, beef protein, and fiber intake	Total BaP binding by microcapsules[b]	Colonic nuclear aberrations, %[c]	Total BaP binding by microcapsules[b]	1,6-Dione trapped in microcapsules[d]
LF/LB/LNSP	60	3.60 ± 0.25	0.93 ± 0.41	3.9 ± 2.2
HF/LB/LNSP	65	1.30 ± 0.35	ND	ND
LF/HB/LNSP	86	2.33 ± 0.20	1.04 ± 0.10	13.3 ± 8.8
LF/LB/HNSP	56	1.87 ± 0.31	0.59 ± 0.11	3.6 ± 2.4
LF/HB/HNSP	ND	ND	0.97 ± 0.02	17.2 ± 1.6
Control	ND	0.45 ± 0.09[e]	0.24 ± 0.01[f]	2.3 ± 1.8[f]

Abbreviations: BaP, benzo[*a*]pyrene; H, high; L, low; F, fat; B, beef protein; NSP, nonstarch polysaccharide; ND, not determined.
[a]Rodents treated *per os* with [^{14}C]BaP.
[b]Expressed as pmoles BaP per million microcapsules. C57/B6 mice data from pooled samples (six per group).
[c]Colonic nuclear aberration included apoptotic cells and micronuclei.
[d]Expressed as pmoles BaP 1,6-dione per million microcapsules.
[e]Control mice consumed LF/LB/LNSP diet but were not treated with BaP.
[f]Expressed as pmole BaP 1,6-dione per million microcapsules.

postlabeling, etc., and a second consisting of DNA sequences, e.g., *ras* and *p53* oncogene sections (*8*) for which damage is known to be critical in human GI carcinogenesis.

Both types of target would be damaged during the vigorous acylation reactions used in forming the microcapsule membrane. An approach adopted for the first target type (low molecular weight) is to insert it into the microcapsules after membrane formation. Preliminary work with guanosine-3′-phosphate coupled by carbodiimide into PEI microcapsules (Ciroussel et al., unpublished data) showed, however, that such target-adducted microcapsules yielded virtually no N7-methylguanine after treatment with [$^{14}CH_3$]-labeled NMU or methylmethane sulfonate (MMS). It appeared that the PEI microcapsule nucleophilicity, while so effective in trapping carcinogen electrophiles, was too effective in shielding this target. Consequently, we lowered the residual nucleophilicity of the microcapsule structure (to ~ 1% of PEI structures) by using a poly(vinyl alcohol)-triethylenetetramine structure and then blocking most of the amino functions (*39*).

A better target substance (I) has been synthesized (*38*). While representing the most reactive DNA site dG deoxyribose is replaced by a selectively cleavable diol linker moiety to avoid extensive depurination and/or phosphate hydrolysis with consequent loss of adducted guanine. This has been shown to react with NMU at a rate and giving O^6/N7 product distribution similar to dG (McGinnis et al., unpublished data). Sodium periodate cleavage of the target linker, followed by $NaBH_4$ reduction to give 9-hydroxyethylguanine has been shown and mass spectrometric assay of this cleaved target and several adducts demonstrated (Farmer et al. unpublished data).

With the components of the first target system in place, another task is to incorporate DNA or polynucleotides into microcapsules to provide the second target type. Encouraging progress is being made on this task. The problem of intragastric hydrolytic damage can be avoided by using enteric-coated capsules to contain the microcapsules. Such procedures may provide specific duodenal release or colonic release (*40*), and thereby some discrimination of the GI segment in which microcapsule trapping has occured.

Microcapsule Trapping and Dietary Influences within the Intestinal Milieu

Although many studies have examined substances of potential carcinogenic significance in the GI cavity and the complex interactions therein, there is still the problem that altering one component of diet or microflora usually has complex and mostly unknowable consequences to which an observed effect of tumorigenesis or fecal excretion may not be directly related. A second problem is that experimental work within GI milieu derived from commercial rodent diets produces results totally unrelated to human consumption and which could be related neither to the established dietary etiology of human CR carcinogenesis nor later to human studies. Hence, diets of human foods were designed to provide all eight permutations (*4*;Cummings et al., submitted) of independent, 3-fold alterations in daily intakes of the three established CR risk-altering macrocomponents, namely, fat (*42*), beef protein (*42*), and dietary fiber nonstarch polysaccharide (NSP; *43*).

The set of four low-fat human diets (for which available calorific intake was based on UK human food tables) were found to be well accepted and to give the same bodyweight increases in F344 rats, irrespective of fiber or beef protein changes (Cummings et al., submitted). Almost every biochemical parameter measured having relevance to carcinogenesis was greatly different from those in a chow control group (*5*). [^{14}C]BaP was given by gavage, and a set of systematic alterations by fiber and beef protein was found (Table 2) for *a*) average trapping in microcapsules (three doses given in 48 hr), *b*) the disposition of ^{14}C between cavity contents and microcapsules, and *c*) the pattern of microcapsule-trapped metabolites as assayed by HPLC (*4,5*). A second study with the corresponding high-fat set (Cummings et al., submitted) in F344 rats showed some differences that may be attributable to a fat-fiber interaction. Overall, 3-fold alterations of intake within the normal human range caused 2- to 3-fold alterations in several parameters potentially related to the effective exposure within the GI tract. The specific trapping on microcapsules of total BaP metabolites (nmole per million) was shown to be inversely related to the 3-day fecal weight, *w*

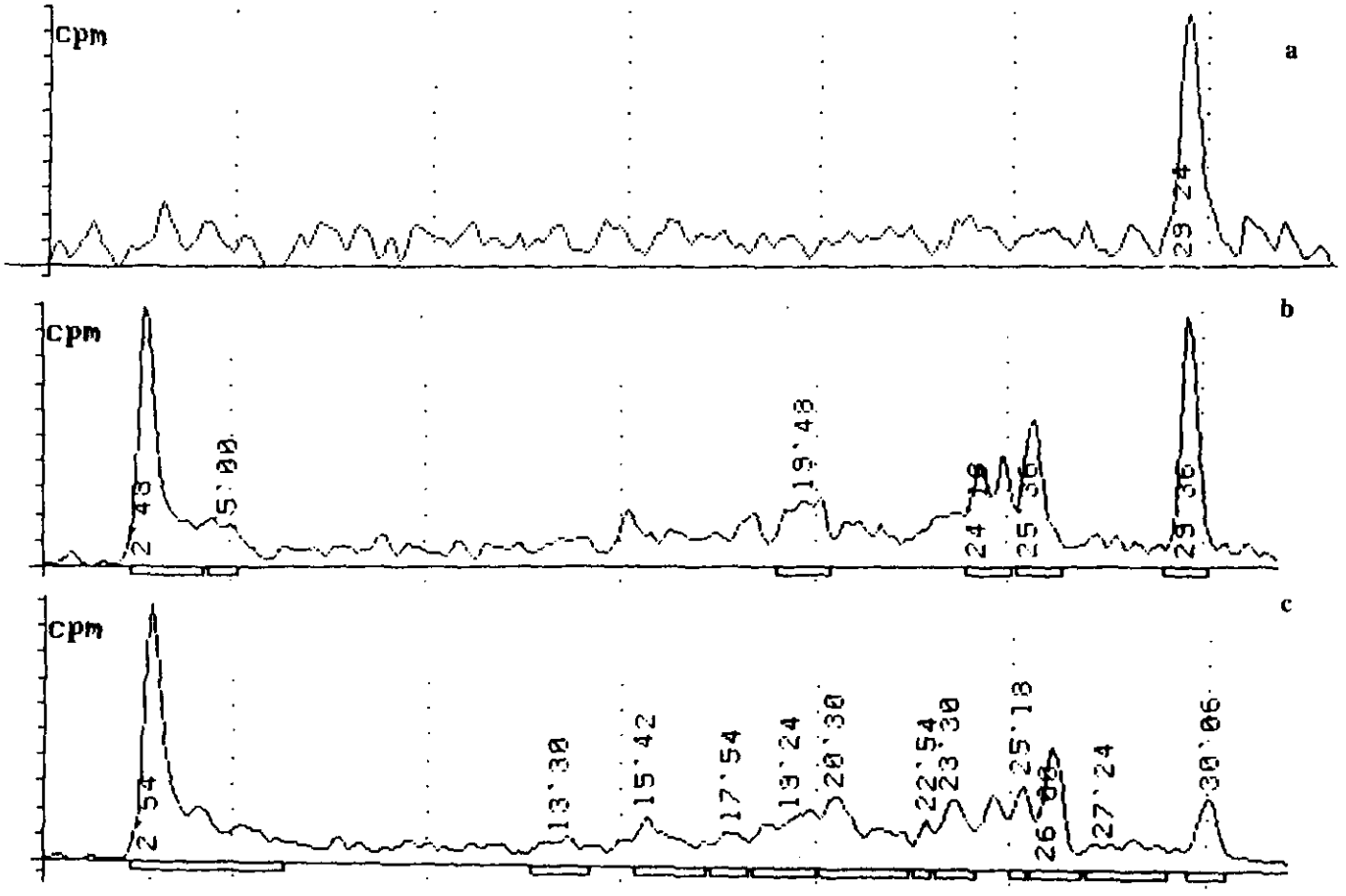

FIGURE 1. HPLC-radiochromatograms for the separation of benzo[*a*]pyrene (BaP) (29.24–30.06 min retention time) and polar metabolites after methanol:NH_4OH (50:1) extraction from microcapsules following gavage treatment of F344 rats with [^{14}C]BaP and PEI microcapsules. Short retention time products ($\lesssim$ 5 min) are (conjugated) BaP tetraols, and those in time 22–27 mins are phenols and diones. Microcapsules were recovered after BaP gavage from (*a*) cecum at 4 hr; (*b*) cecum at 24 hr; and (*c*) feces at 24 hr.

(R=0.81), as one might expect from dilution of intestinal contents (*25*; Cummings et al., submitted). In turn, w was principally dominated by fiber intake (in accord with one hypothesis on fiber action) with w(g) = 0.37 + 0.81 (NSP) + 0.27 (beef protein). These studies of the rodent intestinal milieu have yielded semi-quantitative effects on microcapsule trapping by altering epidemiologically established dietary factors within the normal human intake range. These results indicate that such dietary factors must be controlled in seeking sources of as-yet unidentified, endogenous DNA-damaging agents.

A third study with [^{14}C]BaP in C57/B6 mice (*44*) did not show such striking differences in microcapsule trapping at 24 hr after gavage, the time chosen for sacrifice in order to measure colonic nuclear alterations (Table 2). These showed large diet-dependent differences, as did the pattern of BaP metabolites extracted from the microcapsules (*44*), but the small fraction of BaP dose present in feces (24–39%) gives rise to caution about relative effects of the first-pass BaP metabolites *versus* those transiting later after duodenal absorption and enterohepatic circulation and metabolism. Dietary influences on the pharmacodynamics of BaP or other carcinogens have influenced the diet dependence of both the effective mucosal exposure and this snapshot of microcapsule trapping. Further work (Incaurgarat et al., unpublished data) was undertaken to check the dependence on time, GI location, and diet of microcapsule and mucosal DNA adduct formation by [^{14}C]BaP in F344 rats. As the microcapsules are moved by peristalsis through successive GI locations, and appear in feces, HPLC assay of extracts from microcapsules (Fig. 1) showed that BaP products within the microcapsules are progressively altered. The proportion of desorbable products decreased from 81 to 26% on passing from stomach to feces. Adduct formation within the large bowel was measured at 24 hr after gavage and the mucosal DNA adducts and those formed within microcapsules recovered from the contents of the cavity showed good correlation (r = 0.86, $p < 0.005$).

Diet dependence of microcapsule trapping of both total and individual metabolites has also been shown for [^{14}C]IQ given *per os* (*25;* Turesky, unpublished data). Thus, for an ingested carcinogen, there seems little doubt that carcinogen trapping by microcapsules is strongly influenced by the close proximity to biological events and dietary influences that determine eventual DNA damage in the CR mucosa.

Studies in Humans

With total control of all the dietary consumptions and excretions of volunteers resident in a clinical nutrition suite, our first objective has been the study of the systematic alteration in levels of a sequence of dietary components before proceeding to the comparison of high versus low risk populations. The first study (*6*) showed lack of ill effects from microcapsules and extensive effects as apparently arising from endogenous radical oxidant precursors and cross-linking agents. Our second study is examining the effects of fiber NSP and grilled beef on several microcapsule features discussed earlier. In view of high risk for neoplasms there, we aim also to study the upper GI tract through which microcapsules move rapidly, and the required technical developments are being considered. Separation of effects from microcapsules from exposures in the upper and lower GI tract is currently being studied. A further requirement for microcapsule validation that seems attainable is the use of ethically acceptable indicators of short-term mucosal changes. Currently we can use several microcapsule end points, and we have an exceptional degree of long-term dietary control, without which human studies may be useless. We should be able to extrapolate effects of consumption of the same diets between humans and rodents in which model carcinogens can be used.

Conclusions

For a number of reasons, biomonitoring the GI tract should have importance for the etiology of GI and perhaps other cancers. After overcoming most of the technical problems with microcapsules, we have shown that microcapsules provide trapping of covalently bound and/or desorbable metabolites for all model carcinogens used. Several different end points were found. In conjunction with magnetically maneuvrable substrates that permit easy recovery, clean-up, and total distinction from any endogenous substrate in the GI tract, these microcapsules provide a number of advantages. Manipulation of established dietary risk factors produces substantial alterations in trapping, as might be expected from the as-yet unproven assumption that cavity levels of carcinogen electrophiles will directly alter GI cancer risk.

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